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Identification of 17–18 kDa zona pellucida binding proteins from boar spermatozoa

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Zona pellucida (ZP) binding proteins from boar spermatozoa were compared with antigens recognized by ACR.2 and ACR.3 monoclonal antibodies. The ZP binding proteins of 55, 53, 45 and 38 kDa are identical with various forms of boar acrosin immunologically detected by ACR.2 antibody. The ZP binding proteins of 17 and 18 kDa are recognized by ACR.3 antibody. The N-terminal amino acid sequence of the 17 kDa protein revealed that it is not derived from an acrosin molecule.

Zona pellucida binding protein; Boar sperm cell; Acrosin; Fucose; Amino acid sequence

1. INTRODUCTION

Fertilization is a complex reaction which consists of several consequently occurring steps [1]. Sperm/egg recognition as the first step in the sperm/egg interaction plays a key role in fertilization.

Several ZP binding proteins have been described in mammalian spermatozoa (for review see [2]). A high-molecular form of acrosin has been identified as a major ZP binding protein from boar spermatozoa [3–5]. This protein recognizes and binds fucose and mannose moieties [3–6]. Besides the major ZP binding protein, other proteins binding the zona glycoproteins and also fucose and mannose moieties have been revealed in boar spermatozoa. Proteins of 67, 38 and 17–20 kDa have been described [2–8].

In this report, we describe identification of the boar sperm ZP binding proteins and compare them with several forms of boar acrosin revealed by ACR.2 monoclonal antibody and with antigens recognized by ACR.3 antibody. For further characterization of the 17 kDa zona binding protein, the N-terminal amino acid sequence of this protein was determined.

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Abbreviations: BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulphate; UEA, *Ulex europaeus* agglutinin; ZP, zona pellucida

2. EXPERIMENTAL

2.1. *Preparation of sperm extract and seminal plasma*

Freshly ejaculated boar spermatozoa were centrifuged at 800 × *g* to remove seminal plasma, and sperm pellet was resuspended in PBS containing 2 mM benzamidine (Sigma) and washed twice. The spermatozoa were resuspended in 20% (v/v) glycerol, 30 mM benzamidine and adjusted to pH 3 with hydrochloric acid, extracted at 4°C overnight, centrifuged at 15000 × g, and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The seminal plasma was spun at 15000 × *g* and applied directly to SDS-PAGE.

2.2. *Electrophoresis and Western blotting*

SDS-PAGE was performed on 12% polyacrylamide slab gels under nonreducing conditions [9] and the proteins were transferred onto nitrocellulose sheets [10]. Molecular weight was estimated by comparison with mobility of low-molecular weight markers (Pharmacia) used in a reduced form.

2.3. *Preparation and biotinylation of ZP proteins*

ZP proteins were prepared as described previously [11]. Protein concentration was approximately estimated by absorbance at 280 nm and the purity of ZP glycoproteins was checked by SDS-PAGE. Protein concentration was adjusted to 1 $mg \cdot ml^{-1}$ with PBS and 100 μl of this solution was added to 100 μl of 0.2 M $NaHCO_3$, pH 9, and reacted with 40 μg *N*-hydroxysuccinimidylbiotin (Sigma) in 20 μl dimethylformamide. After 30 min at 25°C, the reaction was stopped by addition of 20 μl 1 M glycine, and the sample was dialysed against PBS and stored at −20°C until used.

2.4. *Reaction of nitrocellulose-immobilized proteins with specific probes*

For immunodetection [10] of ZP proteins, monoclonal antibody 4F2 [11] and for detection of acrosomal proteins, monoclonal antibodies ACR.2 and ACR.3 [12] were used. The ZP binding proteins were revealed either by incubation with biotinylated ZP proteins (stock solution diluted 1:100) or by incubation with nonmodified ZP proteins (100 $\mu g \cdot ml^{-1}$) and then with monoclonal antibody against

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ZP (4F2). The binding experiments in the presence of fucoidan (1 mg·ml^{-1}) were used as a negative control. For identification of fucose-bearing proteins, nitrocellulose was probed with biotinylated UEA (Sigma) at a concentration of 10 μg·ml^{-1}. Biotinylated probes were identified by Streptavidin-peroxidase (1:300, Amersham), using the protocol recommended by the manufacturer. For total protein visualization, the nitrocellulose was stained with colloidal gold solution [13].

2.5. *Purification of the 17 kDa ZP binding protein*

Lyophilized sperm extract (240 μg) was subjected to 12% SDS-PAGE [9] and transferred onto a glass fiber sheet (GF-C, Whatman) coated with Polybrene (Sigma) [14]. Electroblotting was performed in 25 mM Tris, 0.5 mM dithiothreitol, pH 8.4, for 4 h at 85 V [15]. The membranes were stained with fluorescamine (10 mg·l^{-1}, Sigma) in acetone. Proteins were visualized under 254 nm UV light and 17 kDa major ACR.3 antigen was cut from the sheet and subjected to sequencing. Position of the ACR.3 antigens was identified by immunoblotting performed in parallel.

2.6. *Amino acid sequence analysis*

The Edman degradation was carried out in a 470 A Gas Phase Protein Sequencer (Applied Biosystems) according to a modified standard program 03 CPTH supplied by the manufacturer. The content of PTHs in the individual cycles was assayed by HPLC using a Beckman Ultrasphere ODS column.

3. RESULTS AND DISCUSSION

In preliminary experiments, radioiodinated [5–7] or biotinylated [3,4] ZP glycoproteins were used to probe sperm proteins. To avoid partial denaturation of ZP glycoproteins during labelling procedures, we also used nonmodified ZP proteins for binding experiments. No differences in binding of biotinylated and nonmodified ZP glycoproteins to sperm and seminal plasma proteins were observed (Figs 1 and 2). When the proteins of sperm extract were probed, we revealed ZP binding proteins of 55, 53, 45, 38 and 17–18 kDa (Fig. 1c,e). When we compared positions of these proteins after SDS-PAGE and protein transfer onto nitrocellulose with several forms of acrosin distinguished by ACR.2 monoclonal antibody (Fig. 1g,j), we could confirm a suggestion [5] that ZP binding proteins not only of 55 and 53 kDa but also of 43 and 38 kDa are identical with boar acrosin. Acrosin molecules were never immunologically detected in boar seminal plasma. The finding is in good agreement with the ones previously described [5,7]. Using the ACR.3 monoclonal antibody we revealed that ACR.3 recognizes proteins with the same electrophoretical mobility as the ZP binding proteins of 17 and 18 kDa (Fig. 1c,d,e, and Fig. 2c,e,g). These proteins are present in acid-extracted sperm proteins and also in the boar seminal plasma. The binding of solubilized ZP to the sperm proteins can be inhibited by fucoidan or dextran sulphate [3–5] and thus the binding experiments in the presence of fucoidan were used as a negative control (Fig. 1d and Fig. 2f).

It was surprising that acrosin and the 17–18 kDa ZP binding proteins can bind the UEA, a lectin with a specificity to fucose moieties (Fig. 1l). This observation and the fact that acrosin and the 17 kDa sperm proteins

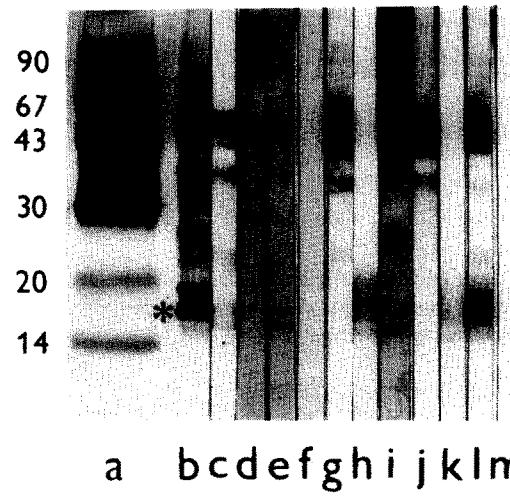

Fig. 1. Reaction of nitrocellulose-immobilized sperm proteins with specific probes. (a) Colloidal gold staining of Pharmacia LMW standards (90, 67, 43, 30, 20, 14 kDa). (b) Colloidal gold-stained total protein. (c) Binding of biotinylated ZP glycoproteins. (d) Binding of biotinylated ZP proteins in the presence of fucoidan. (e) Detection of ZP binding proteins by incubation with ZP proteins and then with 4F2 antibody. (f) Binding of 4F2 antibody only. (g) Binding of ACR.2 antibody. (h) Binding of ACR.3 antibody. (i) Blot was incubated with boar sperm extract (10 μg·ml^{-1}) and then probed with ACR.2 antibody. (j) Detection was carried out under the same conditions as in (i) except that fucoidan was added to the boar sperm extract solution. (k) Detection was performed as in (j) but ACR.3 antibody was used. (l) Detection of fucosylated glycoproteins by biotinylated UEA. (m) Control experiment in the presence of fucoidan.

show fucose-binding activity [3–5], suggest that both molecules bind one another. This suggestion was confirmed by the experiment, in which the nitrocellulose-immobilized sperm proteins were incubated with the

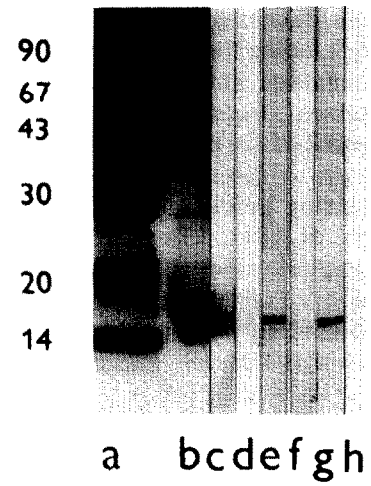

Fig. 2. Reaction of nitrocellulose-immobilized seminal plasma proteins with specific probes. (a) Colloidal gold staining of Pharmacia LMW standards. (b) Colloidal gold-stained total protein. (c) Binding of ACR.3 antibody. (d) Binding of ACR.2 antibody. (e) Binding of biotinylated ZP proteins. (f) Binding of biotinylated ZP proteins in the presence of fucoidan. (g) Detection of ZP binding proteins by incubation with ZP glycoproteins and then with 4F2 antibody. (h) Binding of 4F2 antibody only.

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1        5        10
A Q N L P X R F L X P A I
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Fig. 3. Amino-terminal amino acid sequences in ZP binding protein of 17 kDa. Sequence analysis was confirmed in two independent experiments.

solubilized sperm proteins and the blot was then probed with ACR.2 antibody. New bands in the positions of the 17–18 kDa ZP binding proteins appeared (compare Fig. 1g with Fig. 1i). The source of the newly observed band of 27 kDa was discussed in a previous paper [12]. This protein is recognized by ACR.4 monoclonal antibody and binds boar acrosin. When fucoidan was added to the solution of boar sperm extract, the newly observed bands would disappear and blots probed with ACR.2 and ACR.3 antibodies would show the normal binding pattern (compare Fig. 1j,k with Fig. 1g,h).

To confirm a suggestion that the 17 kDa ZP binding protein is not related to the acrosin, this protein was subjected to amino acid sequence analysis. The sequence of 11 amino acids from the N-terminus (Fig. 3) was compared with SWISSPROT protein sequence library and with a sequence of the preproacrosin gene [17]. Neither acrosin nor another protein had sufficient homology with the N-terminus of the 17 kDa protein.

The findings evidenced in this work suggest that, although acrosin seems to be a major ZP binding protein of boar spermatozoa, at least two distinct families of ZP binding proteins are present in these cells.

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REFERENCES

[1] Wassarman, P.M. (1987) Science 235, 553–559.
[2] O'Rand, M.G. (1988) Gamete Res. 19, 315–328.
[3] Töpfer-Petersen, E. and Henschen, A. (1987) FEBS Lett. 226, 38–42.
[4] Töpfer-Petersen, E. and Henschen, A. (1988) Biol. Chem. Hoppe-Seyler 369, 69–76.
[5] Jones, R., Brown, C.R. and Lancaster, R.T. (1988) Development 102, 781–792.
[6] Jones, R. (1987) Cell. Biol. Int. Rep. 11, 833.
[7] Brown, C.R. and Jones, R. (1987) Development 99, 333–339.
[8] O'Rand, M.G., Matthews, J.E., Welch, J.E. and Fischer, S.J. (1985) J. Exp. Zool. 235, 423–428.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[11] Kyurkchiev, S.D., Surneva-Nakova, T.N., Ivanova, M.D., Nakov, L.S. and Dimitrova, E.H. (1988) AJRIM 18, 11–16.
[12] Pěknicová, J. and Moos, J. (1990) Andrologia, in press.
[13] Moeremans, M.G., Daneels, G. and Mey, J.D. (1985) Anal. Biochem. 145, 315–321.
[14] Vanderckhove, J., Bauw, G., Puype, M., Van Damme, J. and Van Montagu, M. (1985) Eur. J. Biochem. 152, 9–19.
[15] Aebersold, R.H., Teplow, D.B., Hood, L.E. and Kent, S.B. (1986) J. Biol. Chem. 261, 4229–4238.
[16] Adham, I.M., Klemm, U., Maier, W.M., Hoyer-Fender, S., Tsaonsidon, S. and Engel, W. (1989) Eur. J. Biochem. 182, 563–568.